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Biological active compounds from *Betula megrelica* grown in georgia

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Abstract
Phytochemical investigation of the bark of *Betula megrelica* Sosn. grown in Georgia led to the isolation and identification of eleven biologically active compounds from ethanol extract. Their structures were determined by NMR, MS, UV and IR methods. The crude extract and enriched fractions from the bark of *B. megrelica*, were studied *in vitro* experiments to reveal their anti-inflammatory, anti-oxidant and anti-cancer activities.

Keywords: Betula megrelica, spectral identification, phenylethanoid

1. Introduction
Gen. *Betula* L. (*Betulaceae*) in Georgian flora is presented with 7 varieties. Among of them, *Betula megrelica* Sosn. (Figure 1.) Is an endemic species [1]. Gen. *Betula* L. is known for its rich constituent of biological active compounds [2-5]. Anti-cancer activity of Betulinic acid has previously reported [6] the arilbuthanoids, diarilheptanoids, lignans and phenolic compounds, possessing high antioxidant, cytotoxic, anticancer and antiviral activities, are isolated from the different species of *Betula* L. [7-11].

2. Materials and methods
2.1. Plant material
The Bark of *Betula megrelica* L. was collected at north side of Mt. Migaria (Georgia). A herbarium specimen (No 7817) is deposed in the herbarium of the department of pharmacobotany, at the TSMU Iovel Kutateladze Institute of Pharmacochemistry (Herbarium TBPH, Tbilisi, Georgia).

2.2. Experimental procedures
2.2.1. Extraction and Isolation
The barks were extracted with ethanol 95% at room temperature, then at 70^0C with ethanol 70% (2 times). The pulled ethanol extracts were evaporated to dryness under reduced pressure. The obtained residue was repeatedly chromatographed on Diaion® HP-20 and rich fractions (H_2O, MeOH 30% contained compounds 1,2, and 3; MeOH 50% contained compounds 5,6,7,8 and 9; 80% MeOH contained compound 10 and MeOH 100% contained compound 11) were obtained. Finally individual compounds from the fractions were isolated by preparative HPLC. The structures of isolated compounds were determined by ^{1}H, ^{13}C NMR (COSY, HSQC, HMBC) (Bruker Avance 400MHz).
TLC analyses of isolated biological active compounds were carried out on silica gel plates (Silica gel 60 F_{254}, Merck) using the following solvent systems:1) $CHCl_3$-MeOH-H_2O 26:14:3; 2) BuOH-HOAc-H_2O 4:1:5; 3) CH_2Cl_2-MeOH-H_2O 50:25:5. The spots were observed under UV light (254 and 365 nm) and on daylight after treatment with the reagents, the chromatograms were heated to 100-150°C.
The purity of tested compounds were evaluated by TLC and analytical HPLC.

2.3. Chemicals and reagents.
HPLC grade acetonitrile of analytical grade were purchased from Merck & Co. Ultrapure water (for HPLC analysis was obtained from a Millipore Classic purification system.

2.4. Chromatographic instrument and conditions.
HPLC-DAD-MS analyses were performed on an Agilent 1100 series HPLC-DAD-MS system. The UV spectra were recorded from 190 to 400 nm. The chromatographic separation was

achieved using an Eclipse XDB-Phenyl column C-18 (4.6 x 250 mm; 5 µm). Mobile phase was composed of water and acetonitrile (Gradient elution); with a flow rate of 1 ml/min and injection volume of 5 µl and all separations were performed at 20°C. An Agilent mass selective detector (VL model) equipped with an atmospheric chemical ionization source (APCI) was employed for MS detection. All mass spectra were acquired in the positive ion mode. The full scan mass spectrum was recorded over the range of m/z 100—1000 with the fragmentor at 70 V. Temperature of the drying gas (N_2) was 350 °C with a gas flow rate of 10 L/min and a nebulizing pressure of 40 psi. The ionisation voltage was 4000 V and the corona current was 15 µA.

2.5. Human cancer cell lines and culture conditions.
The human lung carcinoma (A-549), colon adenocarcinoma (DLD-1) and normal skin fibroblast (WS-1) cell lines were provided from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in Dulbecco's minimum essential medium (DMEM) with Earle's salts. A 10% fetal bovine serum, a solution of vitamins, sodium pyruvate and non-essential amino acids (all at a 1:100 v/v dilution of supplied solutions), penicillin (100 IU/ml) and streptomycin (100mg/ml) were added to the culture medium. Cells were maintained at 37 C in a humidified environment containing 5% CO2 (Legault and Pichette, 2007) [11].

2.6. Cytotoxicity by resazurin assay
The cytotoxic activity of extracts and enriched fractionsobtained from wild-grown flowers, wild-grown leaves and *in vitro*-grown leaves was analyzed using resazurin on an automated 96-well Fluoroskan Ascent F1TM plate reader (Labsystems) as described by O'Brien *et al*. [20]. Etoposide was used as a positive control. Cytotoxic activities of tested extracts were showed as means ± standard deviation and indicate the concentration inhibiting 50% of cell growth (IC50). Each study was carried out three times in triplicate.

2.7. Cytotoxicity by Hoechst assay
After reading the resazurin test, cells were treated for cellular DNA assay with Hoechst dye 33342. After washing with PBS solution, dried cells were kept at -80 °C until the Hoechst assay was carried out [21]. Cytotoxicity data were represented by using means ± standard deviation and expressed by the concentration that inhibited 50% of cell growth (IC50). Each study was carried out three times in triplicate.

2.8. ORAC assay (oxygen radical absorbing capacity)
The ORAC values of wild-grown flower, leaf and *in vitro*-grown leaf extracts were measured as described by Ou *et al*. (2001). ORAC values were illustrated in mmoles of Trolox equivalents (TE) per gram (mmol TE/g).

2.9. Antioxidant cell-based assay using 20'.7' - dichlorofluorescindiacetate (DCFH-DA)
The cellular antioxidant activity of wild-grown flower, leaf and *in vitro*-grown leaf extracts was demonstrated using the DCFH-DA assay as designated by Legault *et al*. (2003). Quercetin and trolox were used as reference compounds. The IC50 was calculated using the logarithmic regression of the dose-response curve. In all tests, the coefficients of detection of the regression (R^2) was higher than 0.95. The IC50 value of the tested extracts was calculated as the mean ± standard deviations of three different tests.

3. Results & Discussion
The phytochemical investigation of the bark of *Betula megrelica* was realised for the first time. On the base of various chromatographic techniques, from the crude ethanol extract of the bark of *B. megrelica* by preparative HPLC, were isolated 11 individual compounds.
Their structures have been determined on the base of Mass-spectral, NMR (1D and 2D) spectral evidences. NMR data of all compounds were in agreement with the data reported in the literature [12, 13].

Compound 1. The NMR spectra were acquired in CD_3OD. The structure was determined as 2-*O*-(α-l-arabinofuranosyl-(1→6)-β-glucopyranosyloxy)-4-(4-hydroxyphenyl) butane. The stereochemistry was deduced from the ^{13}C NMR data which were compared to those of ref. [12] and [16]. This conclusion was further supported by an acid hydrolysis yielding l-arabinose and d-glucose as demonstrated by chiral GC. Since the measured specific optical rotation (−68.5, *c* 0.3, MeOH) and the reported one (−85.3 in ref. [12]) are both levorotatory, it can be concluded that 5 is 2*R*-*O*-(α-l-arabinofuranosyl-(1→6)-β-d-glucopyranosyloxy)-4-(4-hydroxyphenyl) butane. Moreover, the acid hydrolysis yielded (−)-rhododendrol as for *compound* 1.

Compound 2-3. Spectra of both compounds were recorded in CD_3OD. Analysis of 1D and 2D spectra allowed to assign their structure as 2*R*-*O*-(α-d-apio-d-furanosyl-(1→6)-β-d-glucopyranosyloxy)-4-(4-hydroxyphenyl)butane and 2*S*-*O*-(α-d-apio-d-furanosyl-(1→6)-β-d-glucopyranosyloxy)-4-(4-hydroxyphenyl)butane, respectively. The stereochemistry was confirmed by NOESY spectra, as well as a comparison of the ^1H and ^{13}C NMR data with those reported by Pan in 1994 [17]. An acid hydrolysis yielded d-glucose further confirming the identification. It is noteworthy that the compounds were separated by about 1 minute in HPLC. The specific optical rotations were measured for *compound* 2 (−80.7, *c* 0.6, MeOH) and *compound*3 (−54.4, *c* 0.09, MeOH) which correspond well with the reported value (−95.5 in [12] and −59.5 in [16], respectively.

Compound 4. The NMR spectra of *compound* 4 were recorded in CD_3OD. The structure was assigned as 1,7-bis(4-hydroxyphenyl)-5-(β-apiofuranosyl-(1→6)-β-glucopyranosyloxy)heptan-3-one. It was already identified from *Betula pendula* [12]. The relative configuration, especially at position 5, has been determined from the excellent ^{13}C NMR data correspondence with those reported in the literature. Furthermore, *compound* 4 was shown to be levorotatory (−29.0, *c* 0.3, MeOH) like the previously reported of value (−50.0 in [12]). *compound* 4 was thus identified as (5*S*)-1,7-bis(4-hydroxyphenyl)-5-(β-apiofuranosyl-(1→6)-β-d-glucopyranosyloxy) heptan-3-one.

Compound 5. The NMR spectra of *compound* 5 were recorded in CD_3OD. Analysis of those ^1H and 2D spectra allowed to assign this molecule as Aceroside VIII, previously isolated from *Betula pendula* [12]. Comparison of the NMR data with those already reported showed that both compounds were identical. Furthermore, *compound* 5 was shown to be levorotatory (−67.1, *c* 1.4, MeOH) like the previously reported of value (−59.1 in [12].
Compound 6. The NMR spectra were acquired in CD_3OD. The structure was established to be 1,7-bis(4-hydroxyphenyl)hept-4-en-3-one [13].

Compound 7. The spectra were recorded in $CDCl_3$. A characteristic 1,4-disubstituted aromatic ring with a hindered rotation was observed, which is the case of the known 3,5'-dihydroxy-4'-methoxy-3',4"-oxo-1,7-diphenyl-1-heptene [13]. In the latter paper, the dextrorotatory compound (+79) was isolated and shown to possess a 3*R* absolute stereochemistry. Since *compound* 7 was also dextrorotatory (+35.0, *c* 0.04, $CDCl_3$), it was formally identified as (3*R*)-3,5'-dihydroxy-4'-methoxy-3',4"-oxo-1,7-diphenyl-1-heptene.

Compound 8. NMR data were recorded in CD_3OD, was easily identified as 4-(4-hydroxyphenyl)-butan-2-ol. The compounds was shown to be levorotatory by a measure of the specific optical rotation (−14.7, c 0.3, MeOH). Since the absolute configuration of (+)-rhododendrol has been established to be 2*S*, *compound* 8 was formally identified as (2*R*)-4-(4-hydroxyphenyl)-butan-2-ol, which is trivially named (−)-rhododendrol [14].

Compound 9. The NMR spectra were recorded in CD_3OD. The spectroscopic data were those of 2-*O*-β-glucopyranosyloxy-4-(4-hydroxyphenyl)butane [14]. With the supposition that glucose was of the d-series, the 2*R* stereochemistry could be inferred, as for compound *compound* 9. The specific optical rotation was measured (−41.5, *c* 0.7, MeOH) and shown almost identical to that of (−)-rhododendrin (reported as −38.0 in [15]). Acid hydrolysis allowed to isolate rhododendrol (−46.9, *c* 0.03, CH_2Cl_2, reported as −17.1 in [15]) and d-glucose. Compound *compound* 9 was thus formally identified as 2*R*-*O*-β-d-glucopyranosyloxy-4-(4-hydroxyphenyl)butane, trivially named (−)-rhododendrin.

Compound 10. Only ^1H and ^{13}C spectra were recorded because of the few amount of 2 available. Comparison of the NMR spectroscopic data with those reported for nudiposide and lyonoside (1) allowed to conclude that *compound* 10 is (7'*R*,8*S*,8'*S*)-9'-(β-d-xylopyranosyloxy)-3,3',5,5'-tetramethoxy-2,7'-cyclolignane-4,4',9-triol, trivially name nudiposide. Especially, the H-7' proton signal of *compound* 10 was observed at 4.09 (d, *J* = 7.5 Hz), while that of 1 was observed at 4.37 (d, *J* = 6.7 Hz), exactly like the reported value [14].

Compound 11. The NMR spectra were recorded in CD_3OD. Unfortunately, the sample was not pure and the few quantity available entailed bad quality spectra. Despite this, resonances characteristic of 1,3,4-trisubstituted aromatic rings and of apiofuranoside, indicated that the compounds could be a diarylheptanoid. However, comparison with literature data didn't allowed a formal identification [19].

The antioxidant, anti-inflammatory and anticancer activities of crude ethanol extracts as well as post-Diaione rich fractions (H_2O, MeOH 30%, 50%, 80% and 100%) from the bark of *Betula megrelica* were evaluated. The MeOH 80% enriched fraction showed a highest antioxidant activity using ORAC method (15.54±1.42 µg/ml) and ethanol extract was the most active in cell based-assay (0.7 ± 0.1 µg/m). The highest cytotoxic activity (IC50) against A-549 lung carcinoma and DLD-1 colon carcinoma cells was detected for enriched MeOH 80% (23 ± 2 µg/ml and 24±1 µg/ml in *Rezazurine* tests) and pure MeOH (23 ± 2 µg/ml and 26±1 µg/ml in *Rezazurine* tests) fractions. The *Hoechst* assays confirmed the same tendency. The enriched MeOH 50% fraction was found the most specific against A-549 lung carcinoma cells with IC50 value of 77 ± 5 µg/ml (>200 µg/ml toward normal skin fibroblast WS-1). The tested samples did not show any significant anti-inflammatory activity.

3.1 Tables and Figures

Fig 1: *Betula megrelica* Sosn.

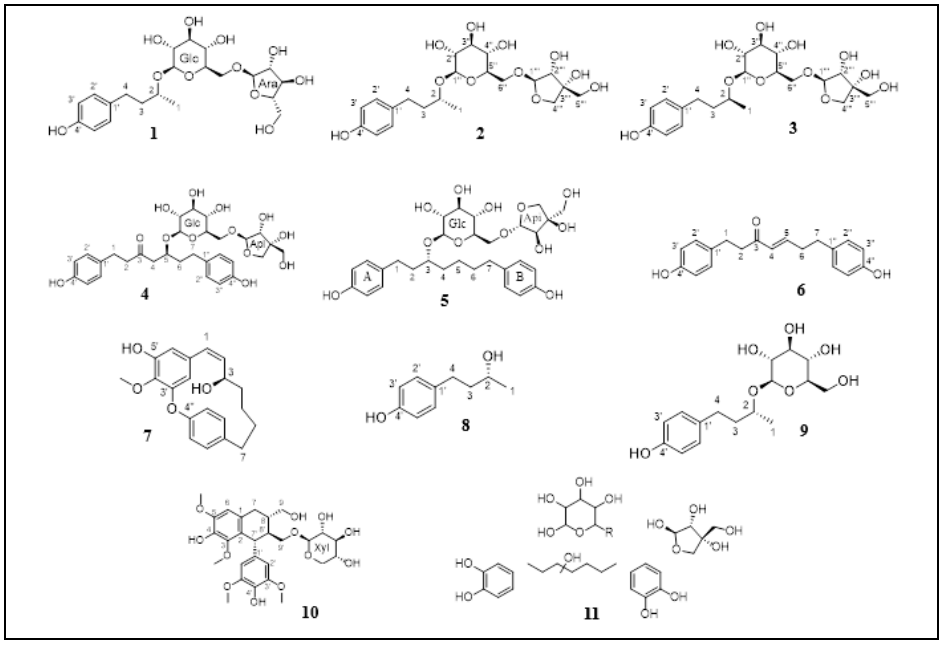

Fig 2: Structure of compounds isolated from *Betula mengrelica*

Table 1: Biological activities of crude ethanol extract and enriched fractions from the bark of *Betula megrelica*

	A-549	DLD-1	DLD-1	WS-1
B. megrelica EtOH extract	160 ± 12 µg/ml	147 ± 15 µg/ml	100 ± 14 µg/ml	70 ± 20 µg/ml
H_2O fraction	>200 µg/ml	>200 µg/ml	>200 µg/ml	>200 µg/ml
MeOH 30% fraction.	>200 µg/ml	>200 µg/ml	120 ± 15 µg/ml	165 ± 47 µg/ml
MeOH 50% fraction.	109 ± 12 µg/ml	139 ± 20 µg/ml	141 ± 12 µg/ml	>200 µg/ml
MeOH 80% fraction.	23 ± 2 µg/ml	24 ± 1 µg/ml	22 ± 2 µg/ml	29 ± 4 µg/ml
MeOH 100% fraction	23 ± 2 µg/ml	26 ± 1 µg/ml	20 ± 2 µg/ml	52 ± 14 µg/ml
Étoposide	1,4 ± 0,2 µM	6,0 ± 0,4 µM	1,6 ± 0,2 µM	9 ± 3 µM

	Antioxydant on WS-1(µg/ml)	**ORAC µmol Trolox / mg**		**Anti-inflammatory % of inhibition (µg/ml)**	**Toxic from**
B. megrelica EtOH extract	0,7 ± 0,1	7.32	±0.14	0,3% (40)	80
H_2O fraction	47 ± 9	0.39	± 0,08	24,9% (40)	80
MeOH 30% fraction.	1,6 ± 0,1	12.33	± 2,89	12,5% (80)	160
MeOH 50% fraction.	2,9 ± 0,1	10.2	± 0,37	11,7% (40)	80
MeOH 80% fraction.	2,0 ± 0,2	15.54	± 1,42	25,2% (40)	80
MeOH 100% fraction	3,2 ± 0,4	8.71	± 0,45	Toxic	
Trolox	0,11 ± 0,01	3.88	± 0,09	L-NAME 250 µM	33±4%
Quercetin	0,23 ± 0,03	20.42	± 2,93	L-NAME 1 mM	68±3%

4. Conclusions

Eleven biologically active compounds were isolated for the first time from the bark of a Georgian endemic species - *Betula megrelica*, and chemically have been fully assigned.

The crude extract and enriched fractions from the bark of B. *megrelica*, were studied *in vitro* experiments for their anti-inflammatory, anti-oxidant and anti-cancer activities.

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